Chapter 4

Noninvasive *In Planta* Live Measurements of H_2O_2 and Glutathione Redox Potential with Fluorescent roGFPs-Based Sensors

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Abstract

In this protocol, we present a noninvasive *in planta* bioimaging technique for the analysis of hydrogen peroxide (H_2O_2) and glutathione redox potential in adult *Arabidopsis thaliana* plants. The technique is based on the use of stereo fluorescence microscopy to image *A. thaliana* plants expressing the two genetically encoded fluorescent sensors roGFP2-Orp1 and Grx1-roGFP2. We provide a detailed step-by-step protocol for performing low magnification imaging with mature plants grown in soil or hydroponic systems. This protocol aims to serve the scientific community by providing an accessible approach to noninvasive *in planta* bioimaging and data analysis.

Key words Noninvasive bioimaging, Fluorescent biosensors, Hydrogen peroxide, Glutathione redox status, Image analysis, Data analysis pipeline, Salt stress

1 Introduction

Plants, being sessile organisms, have evolved extensive mechanisms for acclimation and adaptation. Whereas the perception of external stimuli is mainly mediated by a battery of receptors, downstream, different second messengers are involved in the transduction of the signal and its amplification [1]. In plants, among second messengers, the role of Ca^{2+} and reactive oxygen species (ROS) has been largely established [2]. In particular, ROS were initially considered only as by-products of aerobic metabolism but their role in signaling is now recognized [3]. Among ROS, hydrogen peroxide (H_2O_2), due to its chemical properties such as its relatively mild reactivity with a relatively long lifetime (from ms to s), plays a prominent role in signaling [3–5]. The recent discovery of the leucine-rich-repeat receptor kinase, hydrogen-peroxide-induced

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Ca^{2+} increases (HPCA1), and the cytosolic thiol peroxidase PRXIIB as respectively extracellular and intracellular H_2O_2 receptors has further certified the role of this molecule in both extracellular and intracellular signaling in plants [6, 7].

The photosynthetic process and photorespiration are the main sources of ROS and H_2O_2 in physiological conditions [3, 8], and a battery of ROS scavengers keep these molecules under a strict control [5]. However, when plants are challenged with both biotic and abiotic stimuli, ROS generators like the plasma membrane NADPH oxidases of the RBOH family are activated and a ROS burst can occur [9]. To cope with such a ROS burst, plants use ROS scavengers whose activity relies on the availability of reducing power. The plant antioxidant machinery includes enzymatic components such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and nonenzymatic antioxidants like ascorbic acid (AA), reduced glutathione (GSH), α tocopherol, carotenoids, flavonoids, and the osmolyte proline [5]. Many antioxidant compounds are simultaneously present in oxidized and reduced states in the form of active redox couples [5]. One of the main redox couples of a plant cell is the oxidized/reduced glutathione (GSSG/2GSH), which is kept reduced by the electrons donated by Nicotinamide adenine dinucleotide phosphate (NADPH) and in turn, for example, can reduce the dehydroascorbate to ascorbate (DHA/ASC) [5]. NADPH is generated mainly via photosynthesis and by different types of NADP-dehydrogenases such as NADP-glyceraldehyde-3-phosphate dehydrogenase, NADP-isocitrate dehydrogenase, NADP malic enzyme, and the enzymes of the oxidative part of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (reviewed in [10]), When plants are challenged with stress, ROS are thus efficiently scavenged by the antioxidant batteries that will be consequently oxidized. However, if the antioxidant batteries are not sufficient to scavenge entirely the produced ROS their cellular level can increase.

Based on these premises it becomes evident that measuring ROS, and specifically H_2O_2, as well as the effects on the redox status of the redox couples are important parameters to gather information about the physiology of the plants in control and stress conditions. Traditionally, biochemical analytical analyses have provided tools to quantify ROS (including H_2O_2) production, redox status of different redox couples, and their total amount [11]. However, these techniques are destructive, require tissue manipulation and homogenization, and thus, possibly also the introduction of artifacts. Moreover, these techniques hardly provide cellular and subcellular resolution [8, 11]. In the last 15 years in vivo imaging based on the use of plants expressing genetically

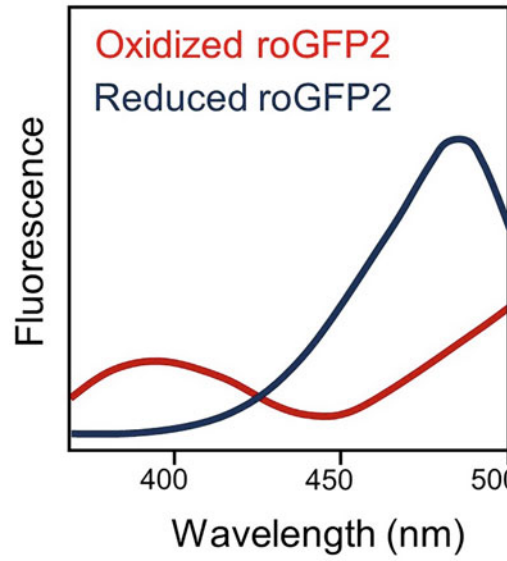

Fig. 1 The spectral properties of purified roGFP2-Orp1. The figure is based on original traces reported in [25], showing the excitation spectra of a purified roGFP2-Orp1 in its fully oxidized (red trace) and fully reduced (dark blue). The isosbestic point is at 422 nm and represents the redox insensitive point [24]

encoded ROS and redox sensors has been established as a powerful and noninvasive technique [11–13]. In particular, redox green fluorescent protein (roGFP)-based sensors have been designed with the intention of measuring both the redox potential of glutathione pool and the H_2O_2 synthesis [12, 14, 15]. Basically, these sensors are based on a modified GFP, where two cysteine residues have been inserted in adjacent β-strands on the surface of the protein β-barrel [11, 16, 17]. When oxidized, these two cysteines form a disulfide bond that induces a structural conformational change that influences the chromophore fluorescence and affects the quantum yields (QY) of the two main absorption peaks (at 405 and 488 nm, respectively) in an opposite way, leading to a ratiometric response [12]. Importantly, the emission spectrum of the sensor remains the same (Fig. 1).

In the cellular environment, roGFPs are in a redox potential equilibrium with the GSSG/2GSH couple, in a reaction catalyzed by glutaredoxin (Grx) (reviewed in [11]). Both the GSH pool and Grx are present in the cytosol and subcellular compartments [11]; however, since in vivo, the roGFP equilibrium with the GSSG/2GSH couple is mediated by Grx, to overcome any possible limitation of Grx availability, the human Grx1 was fused to the roGFP2, making a 1:1 proportion between the roGFP2 sensor and the catalyst [14]. *Arabidopsis thaliana* (hereinafter *Arabidopsis*) plants expressing the Grx1-roGFP2 targeted at the cytosol and different subcellular compartments are available (Table 1) [18–22]. In

Table 1
Grx1-roGFP2 and roGFP2-Orp1 lines of *Arabidopsis* for the analysis of the glutathione redox status and H_2O_2

Available sensors	E_{GSH}	H_2O_2
Sensor	Grx1-roGFP2	roGFP2-Orp1
Operator/sensor	Human glutaredoxin 1 (hGrx1)	Yeast glutathione peroxidases like 3 (Gpx3/Orp1)
Subcellular localization		
Cytosol/nucleus	Grx1-roGFP2 (17)	roGFP2-Orp1 (14)
Cytosol	Cyt-GRX1-roGFP2 (18)	roGFP2-Orp1 (Cyt) (21)
Mitochondria	mito-roGFP2-Grx1 (19)	mt-roGFP2-Orp1 (14)
Plastids	Grx1-roGFP2 (Plastids) (20)	roGFP2-Orp1 (plastids) (20)
Peroxisomes	per-Grx1-roGFP2 (18)	roGFP2-Orp1 (Per) (21)
Nucleus		roGFP2-Orp1 (Nuc) (21)
Apoplast		roGFP2-Orp1 (Apo) (21)
Optical characteristics	*Grx1-roGFP2*	*roGFP2-Orp1*
Fluorescent protein	EGFP	EGFP
Excitation maximum	395 nm, 490 nm	395 nm, 490 nm
Suggested excitation wavelength	405 nm, 488 nm	405 nm, 488 nm
Excitation setup	400 nm, 460 nm	400 nm, 460 nm
Emission maximum	511 nm	511 nm
Emission filter	525/36 nm	525/36 nm
Ratio calculation	$400_{exitation}/460_{excitation}$	$400_{exitation}/460_{excitation}$
In vitro dynamic range	12 (390 nm/480 nm)	8 (400 nm/482 nm)
Plant dynamic range	5 (400 nm/ 460 nm)	6 (400 nm/460 nm)

conclusion, the Grx1-roGFP2 is a sensor for the in vivo determination of the redox potential of the glutathione pool.

It has been shown that the Yeast Orp1 peroxidase has an intrinsic capacity to act as a specific H_2O_2-dependent protein thiol oxidase when it is recruited into proximity of oxidizable target proteins [23]. By leveraging this property, Gutscher et al. developed the roGFP2-Orp1 where the roGFP2 is subjected to an H_2O_2-dependent oxidation mediated by the Orp1. In this way, the roGFP2 becomes oxidized (formation of the disulfide bridge) specifically in response to H_2O_2 and no other oxidants. Thus, roGFP2-Orp1 is a bona fide H_2O_2 sensor. However, a key aspect

that needs to be considered is that the roGFP2-Orp1 oxidized form is reversibly reduced in vivo, most probably by the Grx and/or the thioredoxins (Trx) systems that directly reduce Orp1 [15]. Because roGFP2-Orp1 undergoes a specific H_2O_2-dependent oxidation, but can also be reduced, its redox state is influenced either by the level of the oxidant (H_2O_2) or by the reductants themselves, such as GSH and thioredoxin [15]. This makes the use of this sensor not completely straightforward for the definition of the H_2O_2 levels [11]. Nonetheless, roGFP2-Orp1 has been successfully expressed in *Arabidopsis* plants, allowing to monitor the H_2O_2 dynamics in different cellular compartments (Table 1) [15, 21, 22]. In conclusion, the roGFP2-Orp1 is a sensor for the in vivo determination of the H_2O_2-dependent oxidation of the roGFP2 sensor.

So far, imaging of *Arabidopsis* plants expressing either Grx1-roGFP2 or roGFP2-Orp1 has been mainly carried out using young seedlings grown in plates or leaf sections from mature soil-grown plants imaged by wide-field and/or confocal fluorescence microscopy as well as by the plate reader. Detailed protocols describing these procedures are available [24, 25]. Remarkably, such microscopy approaches require a certain grade of sample manipulation, provide only a view of a few numbers of cells, and do not provide information at the organismic level. However, Hipsch and colleagues have imaged entire *Arabidopsis* and *Solanum tuberosum* (potato) plants expressing the roGFP2 localized into chloroplasts in physiological conditions using an Advanced Molecular Imager HT (Spectral Ami-HT, Spectral Instruments Imaging, LLC., USA) [26–28].

Here, we present a noninvasive methodological approach based on the use of a stereo fluorescence microscope to study cytosolic glutathione redox potential and H_2O_2 level in adult *Arabidopsis* plant leaves expressing Grx1-roGFP2 and roGFP2-Orp1 sensors. We provide a guideline to perform experiments with plants grown in standard conditions in soil and hydroponics and in response to salt stress treatment. The principles of the approach described here can be generalized to other fluorescent protein sensors such as for pH and Ca^{2+}.

2 Materials

2.1 Sensors, Plant Lines, and Growth Material

1. For the Grx1-roGFP2 and roGFP2-Orp1 sensors, a comprehensive in vitro characterization is available, including a reliable account of their respective spectroscopic characteristics, specificities, and sensitivity ranges [12, 15]. Different constructs for *Agrobacterium*-based plant transformation for stable transgenic lines in *Arabidopsis* Col-0 background are available (*see* **Note 1**) (Table 1 adapted from [25]). Barley seeds expressing the Grx1-roGFP2 have also been obtained [29].

2. Mature *Arabidopsis* Col-0 plants (3–4 weeks) expressing the Grx1-roGFP2 and roGFP2-Orp1 are used for treatments.
3. Plant growth chamber with temperature, lights, and humidity control.
4. MS/2 plates (0.5× Murashige and Skoog medium), pH 5.8 (adjusted with KOH), solidified with 0.8% plant agar.
5. 1 M hydrogen peroxide (H_2O_2) stock solution in deionized water.
6. 1 M dithiothreitol (DTT) stock solution in deionized water.

2.2 Hydroponic Cultures

1. Seed-holders (Araponics, https://www.araponics.com/) mounted on tip boxes.
2. Rockwool.
3. Composition of the hydroponic solution: 1.5 mM $Ca(NO_3)_2$, 1.5 mM KNO_3, 1.5 mM $Mg(SO_4)$, 1.5 mM KH_2PO_4, 1.5 mM $(NH_4)_2SO_4$, 0.1 mM $C_{10}H_{12}FeN_2NaO_8$, 0.15 mM $Na_2O_3Si \times 9H_2O$, 0.05 mM KCl, 0.01 mM $MnSO_4$, 0.0015 mM $CuSO_4$, 0.002 mM $ZnSO_4 \times 7H_2O$, 0.05 mM H_3BO_3, 75 nM $(NH_4)_6Mo_7O_{24}$.
4. As a final concentration, 100 mM NaCl was added to the hydroponic solution.

2.3 Microscopy

1. A stereo fluorescence microscope fits the requirement to carry out the measurements described here. The method described is for Nikon SMZ18 (Nikon Instruments Europe B.V, https://www.microscope.healthcare.nikon.com/en_EU/products/stereomicroscopes-macroscopes/smz25-smz18) (Fig. 2d), with motorized Zoom and filter wheel, but it can be applied to other setups. The microscope was equipped with standard GFP-B/GFP-L/RFP filters and with a custom-built set for roGFP2 ratiometric imaging (Excitation filter 405/20×, dichroic mirror T505lpxr and Emission filter 525/36 nm) (*see* **Note 2**).
2. Excitation light for the sensor is produced by a pE-300ultra CoolLED (CoolLED, https://www.coolled.com/products/pe-300ultra/) with a 400, 460, and 550 nm wavebands configuration to match filter sets within the microscope. The roGFP2 sensors are excited sequentially with two different wavelengths and specifically switching between 460 nm and 400 nm by motorized filter wheel (*see* **Notes 3** and **4**).
3. Images are collected with a Hamamatsu ORCA-Flash4.0 LT Digital CMOS camera (Hamamtsu, https://www.hamamatsu.com/eu/en/product/cameras/cmos-cameras/C11440-42U40.html) (*see* **Note 5**).

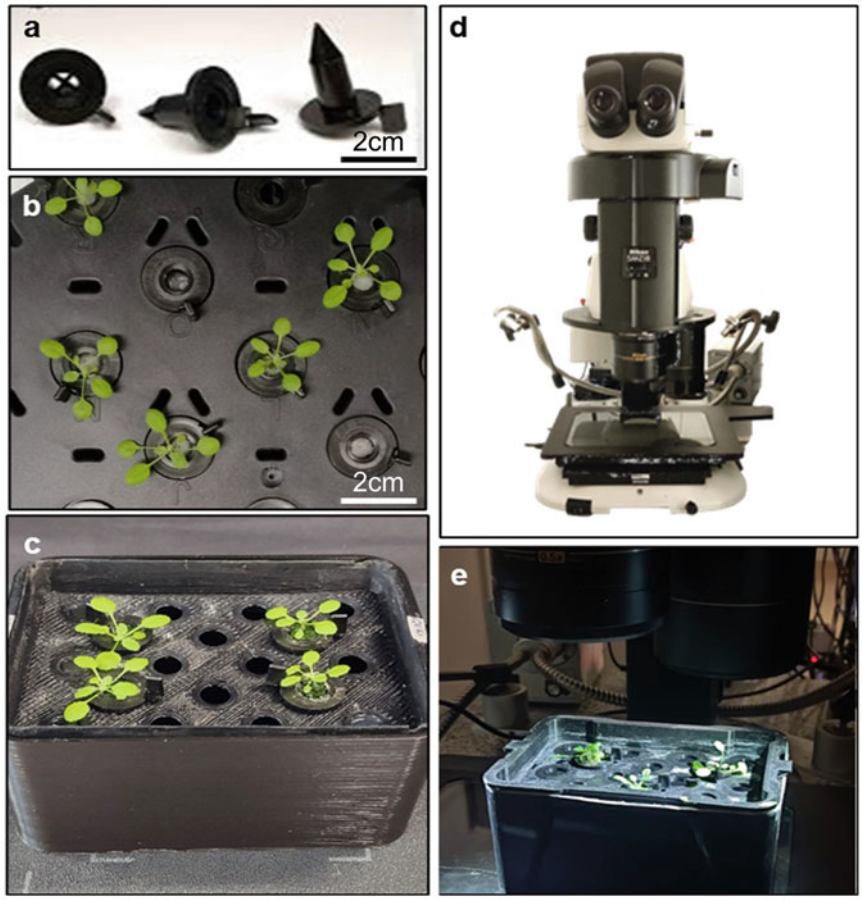

Fig. 2 Experimental setup for in vivo mesoscopic imaging. (**a**, **b**) Plant-holders for plant growth in hydroponic. (**c**) 3D-printed hydroponic system. (**d**) Nikon SMZ18 fluorescence stereo microscope. (**e**) Whole plant imaging for unperturbed in vivo acquisition

4. For roGFP2s analysis, images are collected using a 505/530 nm bandpass filter (GFP) at both excitation wavelengths (460 and 400 nm). The two collected images used for ratio calculation are named 460_{em} (emission 1, 505/530 nm for roGFP2 excited with 460 nm) and 400_{em} (emission 2, 525/36 nm for roGFP2 excited with 400 nm) (*see* **Note 6**).
5. For the largest image acquisition, a 0.5× objective numerical aperture (NA) 0.075 or equivalent is used and zooming can be used (from 0.75 to 13.5×) (*see* **Note 7**).
6. For higher magnifications a 1× objective NA 0.15 can be selected.
7. The NIS-Elements (Nikon Instruments Europe B.V) is used as a platform to control the microscope, illuminator, and camera, and for post-acquisition analyses (*see* **Note 8**).

8. Fiji (https://fiji.sc/) image processing package is installed on PC or Mac (*see* **Note 9**).
9. Ratiometric image analysis is critical to extract qualitative and quantitative data. We use the NIS-Elements (Nikon Instruments Europe B.V), the Fiji software for pixel intensity quantification of 400_{em} and 460_{em} signals in the acquired images (*see* Subheading 3.5).

3 Methods

A procedure is presented here to quantify the glutathione redox potential and the H_2O_2 levels, based on measurements of the $400_{em}/460_{em}$ ratio for Grx1-roGFP2 and roGFP2-Orp1 in non-detached rosette leaves of adult *Arabidopsis* plants in three conditions: soil grown plants, plants grown hydroponically in standard conditions, and plants grown hydroponically in the presence of 100 mM NaCl.

The aim is to illustrate the main steps and considerations for routine measurements.

3.1 Plant Growth and Sample Treatment

1. For soil grown plants, *Arabidopsis* sterile seeds are germinated in MS/2 medium, checked for the presence of fluorescence by using the GFP filter set, and then transferred to pots filled with soil. For homozygous lines, seeds can be directly sown in soil.
2. For hydroponically grown plants, *Arabidopsis* sterile seeds are germinated in MS/2 medium, checked for the presence of fluorescence by using the GFP filter set, and then transferred on Araponics seed-holders filled with Rockwool, and placed in the pipette tip rack located in the tip box filled with the hydroponic solution (Fig. 2a) (*see* **Note 10**).
3. Pots and boxes with seeds are transferred to the growth chamber under long-day conditions (16 h light/8 h dark cycles, 100 Me m^{-2} s^{-1} of cool white neon lamps) at 22 °C and 75% relative humidity and imaged after 3 weeks of growth (*see* **Note 11**). Long-day-grown plants are well-suited to whole-plant imaging due to the relatively small size of the rosette (Fig. 2b, c).
4. Plants grown on soil are watered every 2 days, whereas for hydroponically grown plants solution is replaced weekly (*see* **Note 12**).
5. Live imaging of plants in physiological conditions would require minimal perturbation to the tissues during measurement and performing *in planta* imaging with a stereo microscope (Fig. 2d, e) guarantees to avoid mechanical stress helping to reduce sample manipulation artifacts (*see* **Note 13**).

6. For salt stress treatment experiment, the hydroponic standard solution is replaced with the same solution supplemented with 100 mM NaCl. Plants are then kept for up to 96 h with or without NaCl in the same growth conditions and images are acquired every 24 h.

3.2 Plants Handling

1. Soil- or hydroponically grown plants at the desired time (e.g., 72 h posttreatment with salt) are removed from the growth chamber and directly placed under the stereo macro lens (Fig. 2e).
2. For large images, a 0.5× objective is chosen which allows the full coverage of the plant. A higher magnification of 1× can be also used, but this enables to capture the image of only single leaves.
3. The sensor expression is checked using fluorescent light in combination with 460 nm excitation and GFP filter.
4. The first specimen is used to set up the microscope and to optimize the instrument settings (zooming, focus, and exposure time, *see* Subheading 3.3). Data collection then begins with a separate set of individuals.

3.3 Adjustments for Sensor Imaging

1. Exposure time is routinely settled to 100–1000 ms with 4 × 4 Camera binning for each fluorescence emission (*see* **Notes 14** and **15**). The exposure time is then chosen based on the expression level of the sensor and the zoom factor. With different excitations, different exposure times can be settled, but the same camera binning must be used.
2. Based on our experience the measured pixel intensities expressed as Fluorescence Arbitrary Units (FAU) should provide at least a value of 3000 (considering the maximum of 65,000 grey levels for a 16-bit camera) for the 400_{em} of both Grx1-roGFP2 and roGFP2-Orp1. Keeping exposure time as short as possible avoids sensor bleaching and minimizes photooxidative stress of plants (*see* **Note 16**).
3. Pixel saturation (pixel intensities close to 65,000 FAU) must be avoided as this prevents a correct analysis. This latter case can potentially occur for roGFP2 emissions using higher magnification and zooming. With the 0.5× objective, the amount of emitted light is generally in the linear range of the CMOS sensor.
4. Once set, the settings must be maintained strictly constant for a given experiment (the different tested treatments). Any change of settings will directly impact the results rendering the measured ratio meaningless.

5. Images are acquired every 5 s for a total of 90 s (*see* **Notes 17** and **18**).
6. The experiment is stored in a dedicated folder using the default software extension (*.nd2 with the NIS software).

3.4 Evaluation of the Proper Sensors' Functionality

1. Before performing any experiment, it is important to evaluate the proper ratiometric response of the sensors. This is necessary to check that the hardware is properly configured.
2. Place an adult plant under the stereo microscope and image a single leaf (Fig. 3a).
3. Start performing the image acquisitions as reported in Subheading 3.3 for a maximum of 5–10 min.
4. With a micropipette directly applied to the leaf adaxial side 5 μL of H_2O_2 solutions at different concentrations (10 mM, 100 mM, 1 M) (Fig. 3a).
5. Collect the images for both fluorescence emissions for any given time and save the data.
6. Perform the image and data analysis as reported in Subheading 3.5.

3.5 Data Collection and Quantitative Image Analysis

1. The simplest type of experiment is to monitor for up to 90 s the steady state H_2O_2 and glutathione redox potential in *Arabidopsis* rosette leaves in standard growth conditions using the fixed acquisition settings. Typically, single images are collected for $\geq$3 individual plants *per* treatment and or conditions ($N = 5$ recommended, from independent plants).
2. Of the analyzed Regions of Interest (ROIs), 400_{em} and 460_{em} corresponding to the entire leaf area or entire rosette are used for the ratio (R) calculation ($400_{em}/460_{em}$) (yellow polygons in Figs. 3, 4, and 5). The 400_{em} and 460_{em} fluorescence emissions are background subtracted before ratios are calculated. The background is mainly based on CMOS noise and autofluorescence from soil or hydroponic support. Background fluorescence must be taken for an ROI chosen outside of the leaf but close to the plant (*see* **Note 19**) (red squares in Figs. 3, 4, and 5).
3. The procedure to perform the image analysis for the $400_{em}/460_{em}$ ratio calculation using the Open-Source Fiji software is briefly described. Open a given acquired experiment with its original file extension (e.g., *nd2 of NIS) with the Fiji platform. Fiji will automatically open two series of images, one for the 400_{em} and one for the 460_{em}. Click on the "Analyze" button and in the drop menu select "Tool," and then press the "ROI Manager" button. A new window will appear. By using the drawing tools of the main bar (square, circle, or

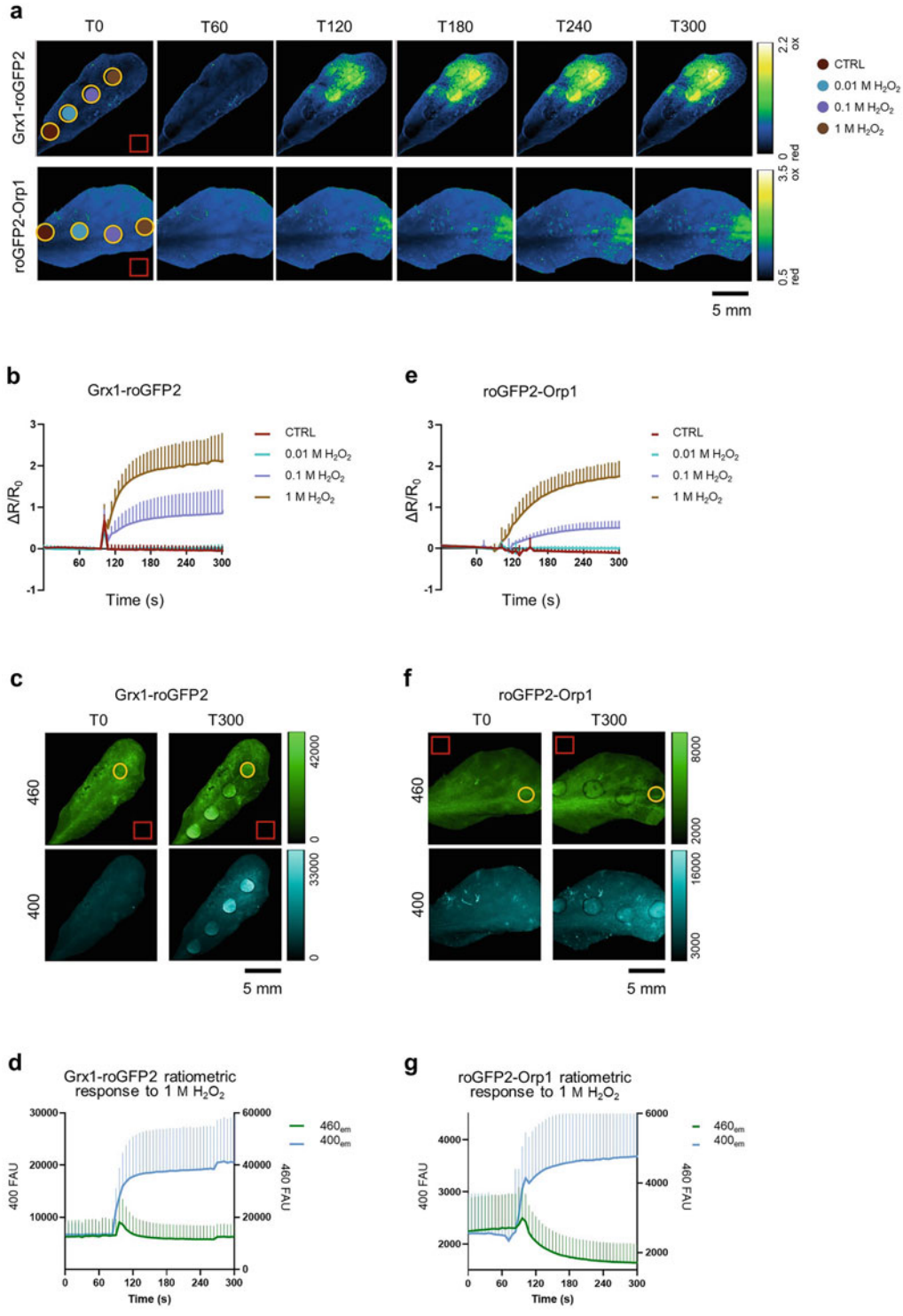

Fig. 3 In vivo H_2O_2-dependent oxidation of roGFP2-based sensors and confirmation of ratiometric response. (**a**) Representative $400_{em}/460_{em}$ ratio images [Green Fire Blue Look Up Table (LUT)] of Grx1-roGFP2 and roGFP2-Orp1 *Arabidopsis* leaves at 0, 60, 120, 180, 240, and 300 s after the start of the acquisition. Treatments (5 μL) from left to right are the following: CTRL, 0.01 M H_2O_2, 0.1 M H_2O_2, 1 M H_2O_2. (**b**, **e**)

polygon) draw an ROI (a circle in Fig. 3a) on the interested region identified in one of the two series of acquired images then on the ROI Manager window press "Add." Repeat the same operation for a background ROI which should be drawn in a region outside the leaf sample. In the ROI Manager window select both ROIs on one of the image series for one of the sensors (e.g., 460_{em} for both Grx1-roGFP2 and/or roGFP2-Orp1) and press the "More" button. In the drop menu select "Multi Measure." A new window will appear, tick the first two options and press "OK." A "Results" list will now open. Click "Results" in the "Results" window, and tick "Mean grey value" in "Set measurements." The values can be copied and directly pasted in an Excel file. Repeat the same routine for the second image series (e.g., 400_{em} both Grx1-roGFP2 and/or roGFP2-Orp1) using the same ROIs added to "ROI Manager."

4. Once 400_{em} and 460_{em} image datasets are reported into the same Excel file, the ratio can be calculated as follows. Subtract the background for each fluorescent channel by subtracting the entire "Mean 2" Column (background) from "Mean 1" Column (sample) indicated in the automatically opened "Results window" from Fiji. The resulting numbers are the pixel fluorescence intensities for 400_{em} and 460_{em} of the selected ROI from which the respective backgrounds have been subtracted. These data series are used for the ratios' calculations:

$$\text{Ratio } 400\text{em}/460\text{em} = (400\text{em} - 400\text{em background})/(460\text{em} - 460\text{em background})$$

5. An average ratio for the 90-s acquisition is then calculated by averaging the ratios for every acquired time point.
6. In the case of Grx1-roGFP2, the higher the $400_{em}/460_{em}$, the higher the oxidation status of the glutathione pool (Fig. 3b).
7. In the case of roGFP2-Orp1, the higher the $400_{em}/460_{em}$, the higher the H_2O_2-dependent roGFP2 oxidation (Fig. 3e).
8. $400_{em}/460_{em}$ for Grx1-roGFP2 ratios can be converted into redox potentials (mV), respectively, through in vivo sensor calibration [12]. In order to do so, R_{max} and R_{min} have to be

Fig. 3 (continued) Normalized ratiometric $400_{em}/460_{em}$ responses of Grx1-roGFP2 and roGFP2-Orp1. $N \geq 5$. (**c**, **f**) Representative false-color images of the two acquired fluorescence emissions of Grx1-roGFP2 (**c**) and roGFP2-Orp1 (**f**) leaves excited with 460 nm (Green LUT) and 400 nm (Cyan LUT) wavelengths, at 0 and 300 s after the application of 5 μL drops of 1 M H_2O_2. (**d**, **g**) Grx1-roGFP2 and roGFP2-Orp1 460 nm and 400 nm emissions changes after 1 M H_2O_2 treatment applied at 100 s. $N \geq 5$. Analyzed Regions of Interest (ROIs) are highlighted by yellow circles in (**c**) and (**f**), background ROIs are highlighted in red. Images were acquired using 0.5× objective lens and 1.5× zoom

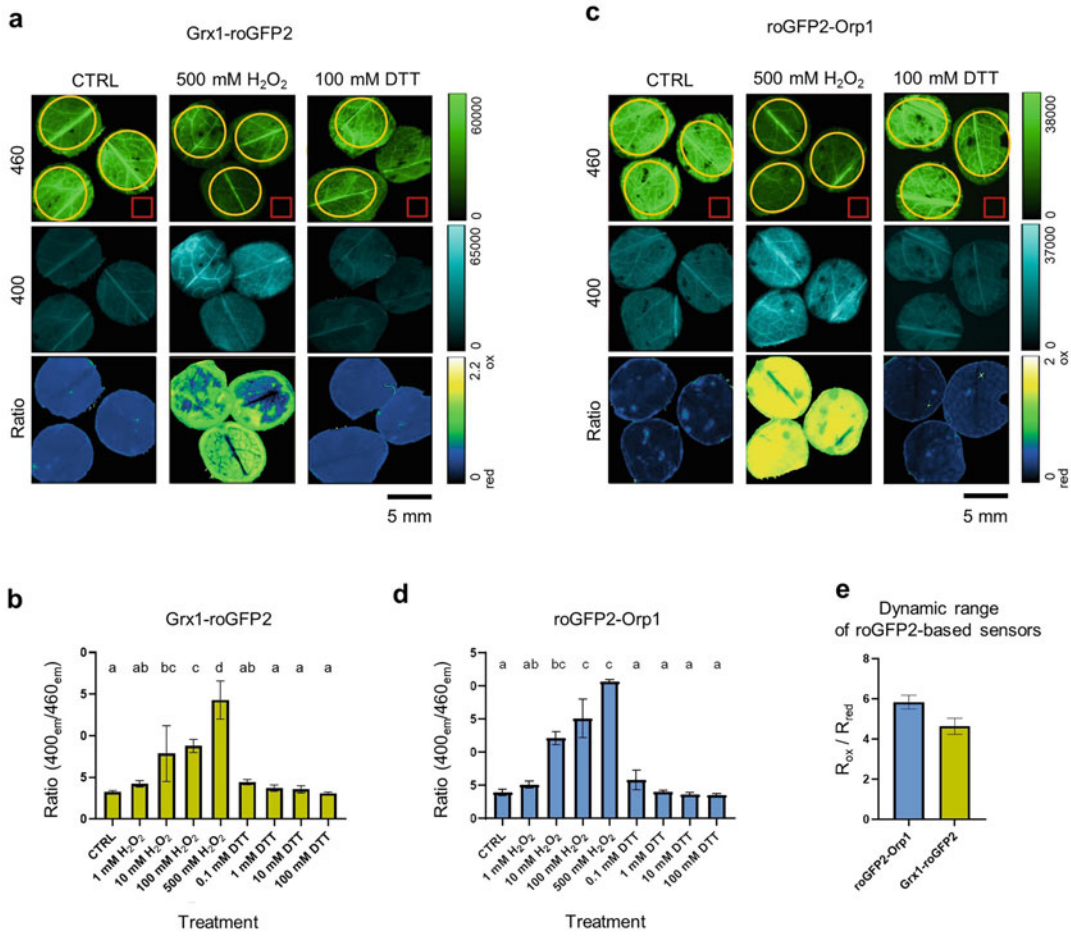

Fig. 4 Determination of in vivo dynamic range of roGFP2-based sensors. (**a**) Representative false-color images of Grx1-roGFP2 leaf disks treated with control (CTRL), 500 mM H_2O_2, and 100 mM DTT solutions for the two acquired fluorescence emissions excited with 460 nm (Green LUT) and 400 nm (Cyan LUT) excitations, and the resulting $400_{em}/460_{em}$ ratio (Green Fire Blue LUT). (**b**) $400_{em}/460_{em}$ ratios of Grx1-roGFP2 leaf disks incubated for 10 min in solutions with different concentrations of H_2O_2 and DTT. (**c**) Representative false-color images of roGFP2-Orp1 leaf disks treated with CTRL, 500 mM H_2O_2, and 100 mM DTT solutions for the two acquired fluorescence emissions excited with 460 nm (Green LUT) and 400 nm (Cyan LUT) excitations, and the resulting $400_{em}/460_{em}$ ratio (Green Fire Blue LUT). (**d**) $400_{em}/460_{em}$ ratios of roGFP2-Orp1 leaf disks incubated for 10 min in solutions with different concentrations of H_2O_2 and DTT. (**e**) In vivo dynamic ranges of roGFP2-based sensors calculated as the ratio between maximum oxidation, obtained by treating the leaf disks with 500 mM H_2O_2, and maximum reduction, obtained by treating the leaf disks with 100 mM DTT. Analyzed regions of interest (ROIs) are highlighted in yellow; background ROIs are highlighted in red. Images were acquired using 0.5× objective lens and 2× zoom. Data are plotted as column bar graphs. Bars represent means and the whiskers indicate the standard deviation. One-way ANOVA with post hoc Tukey's test. $N = 3$. *CTRL* control

measured by treating entire leaves with a concentration of H_2O_2 sufficiently high to fully oxidize the sensor (up to 500 mM) and DTT to fully reduce the sensor (up to 100 mM) (Fig. 4a, c). To improve the efficacy of the treatment

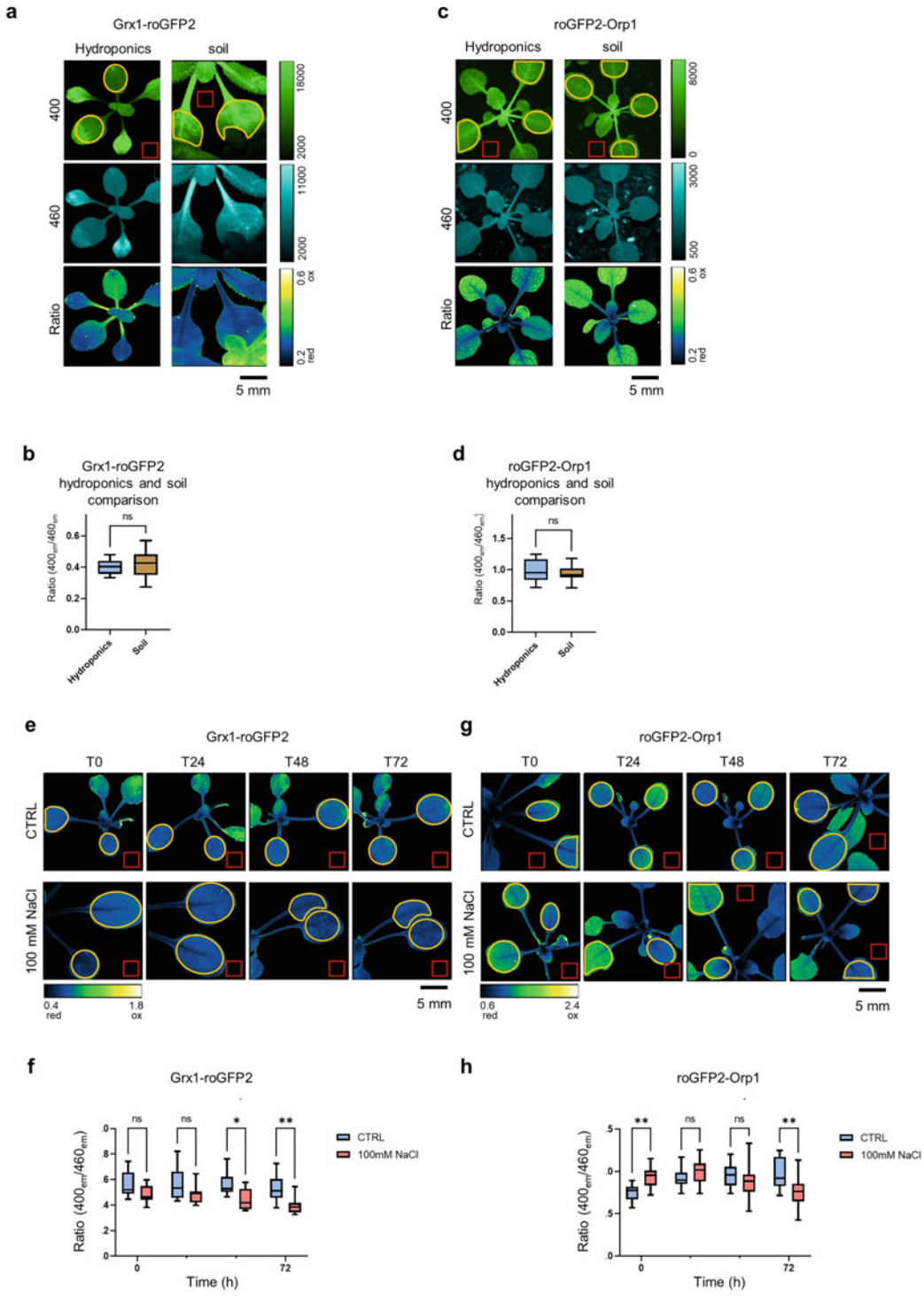

Fig. 5 Case studies: Redox status of Grx1-roGFP2 and roGFP2-Orp1 sensors in adult plants grown in different conditions. (**a**) Representative false-color images for the two acquired fluorescence emissions of Grx1-roGFP2 excited with 460 nm (Green LUT) and 400 nm (Cyan LUT) excitations, and the resulting $400_{em}/460_{em}$ ratio (Green Fire Blue LUT) of plants grown in hydroponics and soil. (**b**) $400_{em}/460_{em}$ ratios of Grx1-roGFP2 plants

leaf discs are obtained from plant sensor lines and dipped for 10 min in solutions containing different concentration of H_2O_2 (1 mM, 10 mM, 100 mM, 500 mM) or the reducing agent DTT (0.1 mM, 1 mM, 10 mM, 100 mM) and then imaged (Fig. 4b, d).

9. Ratio images such as those reported in Figs. 3a, 4a, c, 5a, c, e, and g, for both roGFP2-Orp1 and Grx1-roGFP2 are generated using the "Ratio Plus Plugin" that can be freely downloaded from https://imagej.nih.gov/ij/plugins/ratio-plus.html and installed accordingly to the indications reported in the webpage. Shortly, once the two series of images are open (e.g., 400_{em} and 460_{em}), press the "Plugin" button and select, in the drop menu, the "Ratio Plus" button. A new window will open, and here select as "Image 1" the 400_{em} and select as "Image 2" the 460_{em}, and then press "OK." A new series of images (the ratio images) will open, and now by selecting the Look Up Table (LUT), different colors can be chosen (e.g., Green Fire Blue for $400_{em}/460_{em}$ Figs. 3, 4, and 5). To allow visual comparisons among ratio images, corresponding to the different treatments, it is necessary to uniform their "minimum" and "maximum" ratio values. This is carried out by opening a ratio images with Fiji and then pressing the "Adjust" button which is found in the "Image" drop menu. Then press the "Brightness/Contrast" button and press the "Set" button. Here, the values of the minimum and maximum ratios can be indicated. Repeat the same routine and use the same minimum and maximum values for all the ratio images which need to be compared (*see* **Note 20**).
10. Ratio (R) values at different time points can be normalized to the initial (R_0) by applying the following formula:

$$\Delta R/R_0 = (R - R_0)/R_0$$

Fig. 5 (continued) grown in hydroponics and soil. $N \geq 14$. (**c**) Representative false-color images for the two acquired fluorescence emissions of roGFP2-Orp1 excited with 460 nm (Green LUT) and 400 nm (Cyan LUT) excitations, and the resulting $400_{em}/460_{em}$ ratio (Green Fire Blue LUT) of plants grown in hydroponics and soil. (**d**) $400_{em}/460_{em}$ ratios of roGFP2-Orp1 plants grown in hydroponics and soil. $N \geq 14$. (**e**) Representative $400_{em}/460_{em}$ ratio images (Green Fire Blue LUT) of Grx1-roGFP2 hydroponically grown plants at 0, 24, 48, and 72 h in control conditions and treated with 100 mM NaCl. (**f**) $400_{em}/460_{em}$ ratios of Grx1-roGFP2 plants at 0, 24, 48, and 72 h in control conditions and treated with 100 mM NaCl. $N \geq 9$. (**g**) Representative $400_{em}/460_{em}$ ratio images (Green Fire Blue LUT) of roGFP2-Orp1 hydroponically grown plants at 0, 24, 48, and 72 h in control conditions and treated with 100 mM NaCl. (**h**) $400_{em}/460_{em}$ ratios of roGFP2-Orp1 plants at 0, 24, 48, and 72 h in control conditions and treated with 100 mM NaCl. $N \geq 9$. Analyzed regions of interest (ROIs) are highlighted in yellow; background ROIs are highlighted in red. Images were acquired using 0.5× objective lens and 1.5× zoom. Data are plotted as boxplots, with whiskers indicating minimum and maximum values. Upper and lower boxplot's boundaries define the 75th and 25th percentiles. The line in the middle of the box corresponds to the median. *ns* not significant, $^{*}p < 0.05$, $^{**}p < 0.005$ (one-way ANOVA with post hoc Tukey's test). $N = 3$. *CTRL* control

and plotted versus time (Fig. 3b, e). R_0 is the ratio measured before treatment administration.

3.6 Analyses of Steady-State Glutathione Redox Potential and H_2O_2-Dependent roGFP2 Oxidation in Soil or Hydroponics

1. Grow plants in soil and hydroponics for 3 weeks as described in Subheading 3.1.
2. Replace the hydroponic solution with a fresh solution weekly and water soil-grown plants every 2 days.
3. Move plants to the stereo microscope and adjust the microscope settings.
4. Record the fluorescence of the channels with 460 and 400 nm excitation lights as described in Subheading 3.3 for 90 s and place the plants back in the growth chamber.
5. Collect data and perform image analysis as described in Subheading 3.5.

3.7 Analyses of Steady-State Glutathione Redox Potential and H_2O_2-Dependent roGFP2 Oxidation in Control and Salt-Stressed Plants

1. Grow plants in hydroponics for 3 weeks as described in Subheading 3.1.
2. Replace the hydroponic solution with a fresh solution with 100 mM NaCl. Control plants are kept with standard solution.
3. At time 0, which corresponds to the shift to 100 mM NaCl, move the plants to the stereo microscope and adjust the microscope settings.
4. Record the fluorescence of the channels with 460 and 400 nm excitation as described in Subheading 3.3 for 90 s and place the plants back in the growth chamber.
5. Repeat the image acquisition at 24, 48, and 96 h posttreatment.
6. Collect data and perform image analysis as described in Subheading 3.5.

4 Notes

1. Both binary vectors and *Arabidopsis* plants expressing the Grx1-roGFP2 and roGFP2-Orp1 localized at different compartments are available. Specifically, to what concern plants where Grx1-roGFP2 and roGFP2-Orp1 are localized to cytosol, nucleus, chloroplasts, mitochondria, and peroxisomes have been recently reported by Arnaud et al. [22].
2. This filter set is needed to excite the sensors with 400 nm and collect the fluorescence emitted in the GFP emission window.
3. Based on our direct experience, we chose to excite the sensor at every acquisition first with the 460 nm and then with 400 nm.

The 400 nm excitation might induce a photosensitization of the sensor as previously reported for HyPer sensor [30].

4. The use of other light sources instead of light-emitting diode (LED) can be used. Mercury or xenon incandescent light sources can be used. In this latter case, there is however the need to have proper excitation filters, a filter wheel (to switch the excitation lights) and a shutter. Nevertheless, LEDs have several advantages over incandescent light sources including lower energy consumption, longer lifetime, improved physical robustness, smaller size, and faster switching since they do not require a shutter.
5. CMOS cameras from other companies can be chosen.
6. Different excitation wavelengths are admissible (e.g., 405 and 488 nm) if they are before or after the isosbestic point of roGFP2 (~ 422 nm), respectively [25].
7. Higher magnifications can be used by using the Zoom knob or other lenses.
8. The Open-Source Microscopy Software Micro-Manager (https://micro-manager.org/) can be used instead of a commercial software as a platform for controlling the microscope, illuminator, and camera.
9. Napari (https://napari.org/) can be used as an alternative to Fiji with the ROI Registration plugin (https://www.napari-hub.org/plugins/napari-roi-registration).
10. Make sure that the rockwool was well soaked with the solution.
11. To uniform the treatment, pots can be randomly distributed among standard greenhouse flats.
12. The water status of plants is very important. For hydroponic cultures the level of the solution must be carefully checked. With old plants the high rate of transpiration can alter the level of the solution.
13. Plants subjected to imaging analyses can be transferred to the microscopy facility 1 day ahead the analyses and possibly maintained in condition like the normal growth conditions. A home-made growth chamber using LED light sources can be easily built.
14. The use of low magnification objectives has the disadvantage of allowing a limited amount of light to illuminate the specimen which requires an increased camera exposure time as well as the need to set the Camera binning to 4×4 which entails a lower resolution compared with the use of a 2×2 camera binning. 2×2 camera binning can be used with specimens having a high expression level of the sensor or when imaged at higher magnification.

15. For a precise and reliable quantification of 400_{em} and 460_{em} fluorescent signal intensities (quantified as pixel intensity), signal should be at least three times higher than the background noise. Due to the different quantum yield of the two roGFP2 excitation peaks [12, 15] it is better to use a higher exposure time for the 400_{em} with respect to the 460_{em} fluorescent channels.
16. Note that blue light can induce plant responses mediated by phototropins such as transient cytosolic calcium concentration increase.
17. For the proper measurement of the resting $400_{em}/460_{em}$ ratios, a period of 90 s acquisition is strongly suggested since it allows to verify if the measured ratios are constant or undergo changes/fluctuations during the analysis. Instantaneous and early ratio changes can be due to a transient touch response, or even by the light used to illuminate the sensor. Fluctuations reduce the reliability of ratio measurements.
18. Longer experiments can be performed. The sampling time can be adjusted depending on the experiment. For long-term treatments, it can be changed.
19. To reduce the background signal, the support for the Araponics holder should be black and opaque. The use of 3D-printed support with polylactic acid (PLA) material is an affordable and reliable solution (Fig. 2c).
20. If ratio images have to be compared as shown in Fig. 3a, the minimum and maximum ratio intervals must be the same for all ratio images.

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